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Transcriptional Changes Associated with Lack of Lipid Synthesis in Parasitoids

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Abstract

Phenotypic regression of morphological, behavioral, or physiological traits can evolve when reduced trait expression has neutral or beneficial effects on overall performance. Studies on the evolution of phenotypic degradation in animals have concentrated mostly on the evaluation of resulting phenotypes, whereas much less research has been dedicated to uncovering the molecular mechanisms that underlie phenotypic regression. The majority of parasitoids (i.e., insects that develop on or inside other arthropods), do not accumulate lipid reserves during their free-living adult life-stage and represent an excellent system to study phenotypic regression in animals. Here, we study transcriptional patterns associated with lack of lipogenesis in the parasitic wasp *Nasonia vitripennis*. We first confirmed that *N. vitripennis* does not synthesize lipids by showing a reduction in lipid reserves despite ingestion of dietary sugar, and a lack of incorporation of isotopic labels into lipid reserves when fed deuterated sugar solution. Second, we investigated transcriptional responses of 28 genes involved in lipid and sugar metabolism in short- and long-term sugar-fed females relative to starved females of *N. vitripennis*. Sugar feeding did not induce transcription of fatty acid synthase (*fas*) or other key genes involved in the lipid biosynthesis pathway. Furthermore, several genes involved in carbohydrate metabolism had a lower transcription in fed than in starved females. Our results reveal that *N. vitripennis* gene transcription in response to dietary sugar deviates markedly from patterns typically observed in other organisms. This study is the first to identify differential gene transcription associated with lack of lipogenesis in parasitoids and provides new insights into the molecular mechanism that underlies phenotypic regression of this trait.

Key words: phenotypic regression, lack of lipogenesis, fatty acid synthase, metabolism, gene transcription.

Introduction

Phenotypic regression of morphological, behavioral, or physiological traits is a common process contributing to evolutionary trait dynamics (Fong and Kane 1995; Porter and Crandall 2003) that is frequently observed when a trait is under negative selection or when bearing a trait is selectively neutral (Lahti et al. 2009). Potential molecular mechanisms affecting trait expression include mutation accumulation in the gene underlying a trait, distortions of gene regulatory mechanisms, and deletion of genes or partial genome losses, of which the latter have been observed frequently in endosymbionts (Dale and Moran 2006; Lynch 2006; Maughan et al. 2007). Numerous bacterial endosymbionts show complete trait degradation, for instance, in cell envelope biogenesis, regulation of gene expression, and DNA recombination and repair due to gene losses (Burke and Moran 2011; McCutcheon and Moran 2012). Except for work on regressed eye development in cavefish (Jeffrey 2009), research on trait degradation in animals has exclusively focused on phenotypic effects, leaving the molecular mechanisms underlying reduced phenotypic expression largely unexplored. The increasing availability of genome sequence information should allow for a more precise evaluation of the mechanisms underlying trait regression, particularly in animals (Ellers et al. 2012).

Parasitoids are rapidly becoming model systems for studying the evolutionary and ecological consequences of trait regression. Numerous studies have demonstrated that different parasitoid species do not synthesize lipids *de novo* in their

adult life-stage (Ellers 1996; Olson et al. 2000; Fadamiro and Heimpel 2001; Rivero and West 2002; Giron and Casas 2003; Casas et al. 2003; Lee et al. 2004; for a review, see Visser and Ellers 2008). Although most parasitoids are capable of utilizing dietary carbohydrates to meet immediate energy demands (Eijs et al. 1998; Jervis et al. 2008), the conversion of such carbohydrates to long-term storage in the form of lipids is impaired. Phylogenetic analysis revealed that lack of this essential metabolic trait evolved independently in parasitoids of three different orders (i.e., wasps, flies, and beetles) (Visser et al. 2010). The recurrence of lack of lipogenesis is remarkable because major metabolic pathways associated with sugar and lipid metabolism are typically highly conserved across taxa (Grönke et al. 2005; Turkish and Sturley 2009; Arrese and Soulages 2010) and lipid reserves play a key role in both survival and reproduction. It has been suggested that *de novo* lipid synthesis has become redundant in parasitoids because host manipulation results in increased lipid levels in the host that are subsequently taken up by the parasitoid (Visser and Ellers 2008). Such redundancy would make the lipogenesis pathway prone to phenotypic regression.

The molecular mechanism underlying absence of lipid synthesis in parasitoids has not yet been resolved, but a prime candidate gene to explain the absence of lipogenesis is a lack of activity for the critical gene fatty acid synthase (*fas*) and associated proteins in the fatty acid biosynthetic pathway. *Fas* is a highly conserved gene that is essential for synthesis of palmitic acid, a precursor for various other lipid types (fig. 1). Degradation of *fas* has been associated with the evolutionary loss of lipogenesis in the parasitic fungus *Malassezia globosa* (Xu et al. 2007). Another candidate gene to explain regressed lipid synthesis is acetyl coenzyme A (acetyl-CoA) carboxylase (*acc*), which was shown to cause severely reduced lipid levels or even lethal embryonic effects when deficient in mice (Abu-Elheiga et al. 2001, 2005). In addition, lack of lipid accumulation may be due to disruption of triglyceride synthesis, as was found in mice containing a mutated and rearranged *lipin-1* gene (Csaki and Reue 2010). Such mutations inhibit phosphatidate phosphatase activity, an essential enzyme in the formation of diglycerides prior to triglyceride synthesis (Carman and Han 2006). Lack of lipogenesis in parasitoids could thus result from reduced or inhibited functioning of one or several genes within fatty acid or triglyceride synthesis pathways (fig. 1).

Given the recent completion of its full genome sequence (Werren et al. 2010), the parasitic wasp *Nasonia vitripennis* offers an excellent opportunity to study regulatory and structural genetic changes underlying trait regression in parasitoids. *N. vitripennis* is a generalist parasitoid that can attack the pupal stages of over 60 different fly hosts (Whiting 1967). Through the action of venom injected during oviposition, this species arrests its host's development and is capable of increasing lipid levels of its preferred hosts within the genus *Sarcophaga* (Rivers and Denlinger 1995). During its free-living adult life, *N. vitripennis* feeds on nectar and host hemolymph but does not convert these carbohydrate-rich food sources into stored lipids (Rivero and West 2002).

Here, we aim to unravel the transcriptional profile associated with lack of lipogenesis in the parasitic wasp *N. vitripennis*. First, we validate lack of lipogenesis in *N. vitripennis* using two techniques: 1) We compare lipid levels in sugar-fed and starved wasps at several time points during adult life to show that adult wasps do not accumulate lipids, and 2) we measure incorporation of labeled isotopes in fatty acids using gas chromatography–mass spectrometry (GC-MS) to show that adult wasps do not synthesize lipids. Second, we assess gene transcriptional responses to sugar feeding, focusing on key genes involved in carbohydrate, fatty acid, and glycerolipid metabolism. Using quantitative RT–PCR assays, we compare transcription of 28 key genes between short-term and long-term sugar-fed and starved females of the same age. This study is the first to investigate the transcriptional profile associated with lack of lipid synthesis in parasitoids.

Materials and Methods

Lipogenic Ability at the Phenotypic Level

Strain AsymC of the parasitoid wasp *N. vitripennis* (Hymenoptera: Pteromalidae) was obtained from an existing laboratory culture at the University of Rochester (van den Assem and Jachmann 1999). Insects were kept at a temperature of 25°C, RH 75%, and a light:dark regime of 16 h:8 h. For the experiments, six females were allowed to oviposit on six pupae of the flesh fly *Sarcophaga bullata* (Diptera: Sarcophagidae) (Carolina Biological Supply Company). After emergence from the host pupae, individual females were randomly assigned to treatment tubes. To test whether female *N. vitripennis* lack lipogenesis, lipid levels were measured in four treatments: 1) at emergence, 2) after 3 days of starvation with access to water on cotton wool, and after 3) 3 and 4) 7 days of feeding on a 10% (w/v) sucrose solution. For 8–18 females per treatment, fat content was determined following the method of David et al. (1975), in which solely neutral lipids (triglycerides) are extracted from samples. Females were dried for 5 days at 70°C after which dry weight was determined. Females were subsequently placed individually in a glass tube containing 4 ml of ether. After 24 h, ether was removed and samples washed with fresh ether. Insects were dried for 5 days at 70°C after ether extraction and dry weight determined again.

To trace the fate of isotopic labels, five replicate females per treatment were fed with a 10% (w/v) sucrose solution in water as a control or sucrose solution with added deuterium oxide (Sigma-Aldrich) at 50% (v/v) of total water added. After 7 days, the females were frozen at −20°C until further processing. To compare our findings with a species that accumulates lipids as adults, we used the honeybee *Apis mellifera* as a

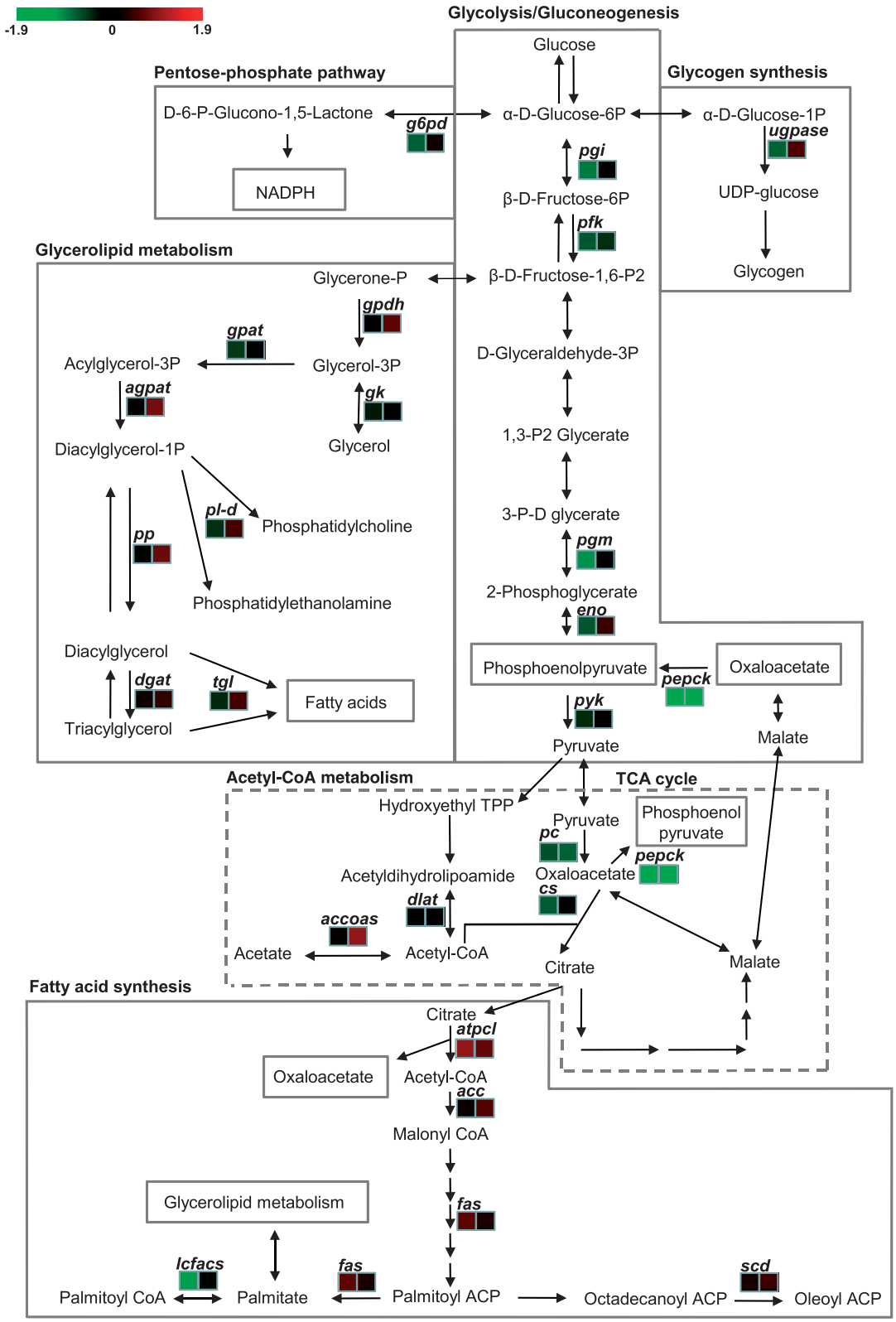

Fig. 1.—Key nutrient metabolic pathways involved in lipid synthesis. Acetyl-CoA metabolism and TCA cycle take place in the mitochondrion (dashed lines); the other pathways take place in the cytosol (solid lines). The conversion of glucose to triglycerides involves three different pathways. Ingestion of glucose first activates the glycolytic pathway that produces pyruvate from glucose. Second, through several enzymatic steps, pyruvate is then converted into

(continued)

positive control. Freshly emerged *A. mellifera* were collected from an existing colony at the USDA-ARS CMAVE Facility, Gainesville, Florida. Insects were kept at a temperature of 20°C, a relative humidity of 40% and in complete darkness. Treatments and number of replicates were similar as described above for *N. vitripennis,* but bees were frozen at −20°C until further processing after 4 days of feeding, a time sufficient for substantial lipid synthesis in young worker bees (Toth et al. 2005; Ament et al. 2011). Lipids were extracted and fractionated for single *A. mellifera* females following the method described by Wessels et al. (2010). Lipid extractions for single *N. vitripennis* were also carried out following the method described by Wessels et al. (2010), but neutral and polar fractions were separated after application of 4 ml of chloroform and 3 ml of methanol, respectively, to silica columns. For both species, thin layer chromatography confirmed that sufficient quantities of solvent were used to fractionate neutral and polar fractions. For GC-MS analyses, only neutral lipid fractions containing triglycerides were used.

To prepare samples for GC-MS, 10 µl of a 1 µg/µl solution of heptadecanoic acid in methylene chloride (Sigma) was added to lipid fractions as an internal standard, after which, samples were dried under a stream of nitrogen. One hundred microliters of methanolic HCl (Supelco) was added and heated for 15 min at 65°C. Methanolic HCl converts all fatty acids, including free fatty acids, di- and triglycerides, within the sample into methylesters. After cooling at room temperature, 1 ml of pentane was added and the vial vortexed for 1 min prior to centrifugation for 8 min at 18,000 × g. The pentane layer was removed for analysis. Routine chemical analyses were conducted using chemical ionization–mass spectrometry (CI-MS, isobutene reagent gas) with an Agilent 5975C MS interfaced to Agilent 7890A gas chromatograph (GC). The GC was equipped with a cool-on-column injector fitted with a 10 cm length of 0.5 mm (i.d.) deactivated fused silica tubing which was in turn connected to 1 m × 0.25 mm (i.d.) length of deactivated fused silica tubing as a retention gap. The retention gap was connected to a 30 m x × 0.25 mm (i.d., 0.25 µm coating thickness) DB5MS analytical column. The conditions of chromatography were: Initial oven and injector temperature = 30°C, 5 min; oven and injector temperatures increased at 10°C/min; final temperature = 225°C. We also obtained total ion spectra (60–500 a.m.u.). Electron impact spectra (60–300 a.m.u.) were obtained using an Agilent 5975B instrument interfaced to a 7890 GC equipped as above, except that the analytical column used was a 30 m × 0.25 mm DB1MS (i.d., 0.25 µm coating thickness).

For analyses, we compared fragmentation patterns and retention times with those of authentic standards. The base peak for straight chain methylesters using CI-MS with isobutane as reagent gas is the result of addition of a proton to the ester resulting in a m + 1 fragment. Thus, although m for methyl palmitate (C16:0) is m/z = 270, the parent ion is m/z = 271. We took this adduct effect into account when assessing mass label incorporation into fatty acids by the insects. Thus, for an addition of 1 deuteron to methyl palmitate, we used abundance of m/z = 272, for m + 2 we used m/z = 273 and so forth. We also analyzed selected samples by electron impact mass spectroscopy to confirm identities of methylesters. For these studies, we used an Agilent 5975B instrument interfaced to a 7890 GC equipped as above except that the analytical column used was a 30 m × 0.25 mm (i.d., 0.25 µm coating thickness) DB1MS®. As in CI-MS studies, retention times and fragmentation patterns were used to confirm identities of natural esters.

Statistical Analyses of Experiments Testing Lipogenic Ability at the Phenotypic Level

The amount of lipid per female was calculated by subtracting dry weight after ether extraction from dry weight before ether extraction. We calculated the percentage of lipids to correct for differences in body size. Normality was inspected using the error structure of the data and homogeneity of variances determined using Levene's test. Data was log-transformed to normality and equal variances. To compare treatments, we used ANOVA followed by a Tukey test to correct for multiple testing.

To analyze isotopic labeling data, we divided the m/z abundance by the C17 internal standard for that sample and calculated the amount of methyl palmitate in nanograms for each sample. For *N. vitripennis*, abundances of m + 4 to m + 6 could not be estimated because the majority of samples were below the detection limit. Normality was inspected using the error structure of the data and homogeneity of variances was determined using Levene's test. We performed *t*-tests to compare the deuterated sugar water treatment with the sugar water control when variances were equal and Welch's *t*-test if

Fig. 1.— Continued

acetyl-CoA. To synthesize fatty acids *de novo* acetyl-CoA is then carboxylated to malonyl CoA by acetyl-CoA carboxylase (ACC), a substrate used by the multidomain enzyme fatty acid synthase (FAS) to form fatty acids through a multistep process. Third, these fatty acids are the raw materials used in the formation of more complex glycerolipids, such as membrane and storage lipids. Sampled genes from pathways other than carbohydrate, fatty acid, and glycerolipid metabolism include AMP activating protein kinase (*ampk*), cGMP-dependent protein kinase (*pkg*), and lipid storage droplet-2 (*lsd2*). Underneath each gene abbreviation, two blocks indicate fold changes between sugar-fed and starved females for the short-term (hours, left block) and long-term treatment (days, right block). Green indicates a gene is down-regulated; red signifies up-regulation. A list explaining abbreviations can be found in table 2.

variances were unequal. All statistical analyses were done using SPSS 14.0

Gene Transcription Experiment

Nasonia vitripennis strain AsymC was obtained from a laboratory culture at the University of Groningen, the Netherlands. The insects were kept at a temperature of 25°C, RH 75%, and a light:dark regime of 16 h:8 h. For the experiments, 20–40 females were allowed to oviposit during 24–48 h on 20 pupae of the blowfly *Calliphora* sp. (Diptera: Calliphoridae) in glass jars sealed with foam stoppers. Jars were inspected daily between 9 and 11AM for newly emerged individuals. Emerged females were randomly assigned to treatments. We applied two feeding treatments: 1) The short-term treatment, in which emerged females were starved for 24 h and subsequently fed for a short-term of 2, 4, 6, or 8 h; and 2) the long-term treatment, in which newly emerged females were immediately fed for 1, 2, or 3 days. Females in feeding treatments were allowed access to water on cotton wool and honey *ad libitum* applied to the foam stoppers. In parallel, we applied two starvation control treatments for the same amount of time, in which females were allowed access to water on cotton wool only. Ten females per treatment were snap-frozen in liquid nitrogen and stored at −80°C for further qRT–PCR analysis. Each treatment consisted of three biological replicates.

RNA was isolated using the SV Total RNA isolation system (Promega) according to the manufacturer's protocol. Successful isolation was confirmed by visual inspection of ribosomal RNA on a 1% agarose gel and RNA quantities were determined using a nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Nanodrop 260–280 nm and 260–230 nm ratios were inspected to assess protein and organic salt contamination. Potential DNA contamination was tested using 1 μl of RNA and a PCR with Taq polymerase, using the primer set of phosphoeolpyruvate carboxykinase (*pepck*), of which the product was run on a 2% agarose gel. Total RNA quantities of clean samples ranged between 50 and 200 ng/μl and were further diluted to a concentration of 50 ng/μl for each sample. cDNA synthesis was done using the M-MLV Reverse Transcriptase system (Promega). cDNA was diluted 8× and stored at −20°C until further processing.

Relevant gene functions were obtained by searching KEGG (Kanehisa and Goto 2000) and orthologs of *N. vitripennis* for metabolic genes of interest retrieved from GenBank. Primers for candidate and reference genes were designed using the program Primer Express 1.5 (Applied Biosystems). Program settings were according to Roelofs et al. (2006). GenBank accession numbers, primer sequences, and efficiencies are listed in supplementary table 1 (Supplementary Material online). In order to determine PCR efficiency, standard curves were obtained in triplicate for the qRT–PCR primer set with 4-fold dilutions of a reference batch of cDNA (Pfaffl 2001). For each qRT–PCR reaction, a total volume of 20 μl was used consisting of 2 μl cDNA template, 10 μl SYBR Green (SensiMix™ SYBR No-ROX kit, Bioline), 1 μl of forward and reverse primer (20 pmol, Eurofins MWG Operon) and 7 μl H_2O. qPCR cycling was performed on a DNA Engine Opticon 1 (Biorad) with three replicates per sample. Cycling program settings were programed according to Roelofs et al. (2006). Reference genes were selected using a pilot dataset consisting of a subset of eight treatments. The pilot included five potential reference genes: elongation factor 1 alpha (*ef1a*), ribosomal protein 49 (*rp49*) (Loehlin et al. 2010), ubiquitin conjugating enzyme (*ubc*), alpha tubulin (*at*) and V-type ATPase (*atpase*), and three target genes: pyruvate kinase (*pyk*), *fas*, and diacylglycerol o-acyltransferase (*dgat*). We used the geNorm analysis application as available in the software package GeNex Light (MultiD Analyses AB) to select the most suitable reference genes. Stable reference genes in our pilot experiment were *ef1a* and *rp49*.

Statistical Analysis of qRT–PCR Data

Opticon Monitor 3 software (Biorad) was used to calculate Cycle threshold (Ct) values. The cycle threshold was set at 0.03 at the beginning of the exponential phase of the curve for all assays. Ct values of three technical replicates were averaged if the standard error percentage did not exceed 20%. If a standard error percentage exceeded 20%, all curves were inspected and the deviating curve removed. For all assays, averages of at least two technical replicate Ct values were used. Ct values were corrected for primer efficiency and normalized based on the formula described by Simon (2003) and using the geometric mean of the two reference genes (Vandesompele et al. 2002). For both short- and long-term treatments, we first fitted a full two-way ANOVA model for each gene, with time, treatment, and time × treatment interaction, and subsequently reduced models by eliminating nonsignificant terms. Except for *atpcl* in the long-term feeding treatment, the minimal explanatory model never retained time as a significant main factor or a significant interaction. We therefore, performed one-way ANOVA with treatment as main factor for all genes. Normality was inspected using the error structure of the data and homogeneity of variances was determined using Levene's test. Non-normal data or data with unequal variances were $\log_2$-transformed. If $\log_2$ transformation did not improve normality or variance, the nonparametric Mann–Whitney *U* test was applied.

Results

Feeding Experiment and Isotope Tracing

Nasonia vitripennis females emerged with an average of 16.3% (±1.1, 1 standard error [SE]) lipids. After sugar feeding, lipid levels declined to 7.6% (±0.7, 1 SE) and 6.9% (±0.8, 1 SE) after 3 and 7 days, respectively (fig. 2). The lowest lipid levels were found in starved females with 4.8% (±0.5, 1 SE)

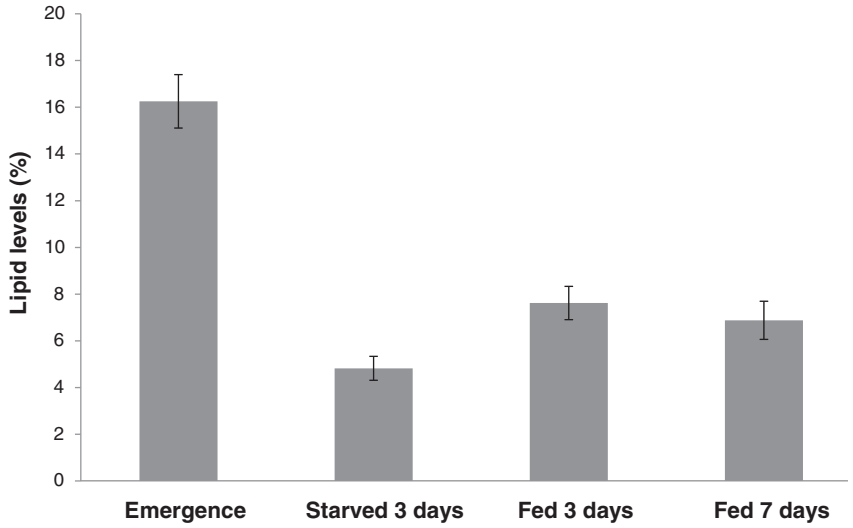

Fig. 2.—Mean percentage of lipids (±1 SE) for *N. vitripennis* females at emergence, 3 days of starvation, and 3 and 7 days after sugar feeding.

Table 1
Results of Isotope Tracing into the Lipid Fraction through Synthesis of Palmitic Acid (C16:0)

	Nasonia vitripennis ($n = 5$ per treatment)				*Apis mellifera* ($n = 5$ per treatment)			
Added Deuterons	Water Mean ng/Sample (1 SE)	Deuterated Water Mean ng/Sample (1 SE)	*t* Value	*P* Value	Water Mean ng/Sample (1 SE)	Deuterated Water Mean ng/Sample (1 SE)	*t* Value	*P* Value
m + 1	210.047 (26.368)	187.707 (39.535)	0.470	0.651	936.773 (50.396)	1165.238 (215.928)	−1.030	0.333
m + 2	22.453 (2.730)	21.559 (4.410)	0.172	0.867	103.344 (5.580)	167.777 (25.980)	−2.425	0.042
m + 3	1.527 (0.427)	1.946 (0.392)	−0.721	0.491	11.076 (2.519)	33.478 (2.775)	−5.977	<0.001
m + 4	—	—			0.108 (0.047)	0.882 (0.074)	−8.807	<0.001
m + 5	—	—			0.260 (0.056)	1.217 (0.491)	−1.937[a]	0.123[a]
m + 6	—	—			0.611 (0.057)	0.825 (0.375)	0.102	0.588

[a]Indicates the result of Welch's *t*-test.

lipids. Lipid levels were significantly different between treatments ($F_{3,53} = 25.728$; $n = 57$, $P < 0.001$), with significantly lower lipid levels after starvation, 3 days and 7 days of sugar feeding compared to females at emergence (Tukey: $P < 0.01$ for all comparisons), thus confirming the lack of lipogenesis in *N. vitripennis* (Rivero and West 2002). We further found that females that had fed on sugar for 3 days had significantly higher lipid levels when compared with females that were starved for a similar duration (Tukey: $P = 0.017$), indicating that *N. vitripennis* females successfully ingested food, leading to a reduced rate of lipid expenditure when sugar was available. When females were fed sugar and deuterated water, no increase in the quantity of isotope labels in palmitic acid was detected compared with females on a sugar control lacking isotopic labels (table 1). Palmitic acid is the main product of fatty acid synthesis and an abundant representative fatty acid in the neutral lipid fraction of *N. vitripennis.* To compare our findings with a species that accumulates lipids as adults, the honeybee *A. mellifera* was fed an identical labeled isotope diet as a positive control. In the non-parasitoid hymenopteran *A. mellifera* significantly increased levels of isotopes were found in palmitic acid, due to *de novo* synthesis of fatty acids (table 1).

Gene Transcription

Transcription levels of 10 out of 28 candidate genes involved in nutrient metabolic pathways were significantly different after short-term feeding compared with starvation (fig. 1 and table 2). Sugar feeding reduced transcript levels for several genes involved in carbohydrate metabolism, including phosphofructo kinase (*pfk*), phosphoglucose isomerase (*pgi*), and phosphoglucose mutase (*pgm*), located at the beginning and end of the glycolytic pathway. Furthermore, the gene UDP-glucose phosphorylase (*ugpase*) involved in glycogen synthesis exerted lower transcript abundance in fed, compared with starved females. Also, two genes involved in the glycolytic pathway and tricarboxylic acid cycle (TCA cycle)

Table 2

Mean Normalized Expression (±1 SE) and Results of Statistical Analyses of Gene Transcription Assays

		Short-term ($n = 24$)				Long-term ($n = 18$)			
Gene		**Fed**	**Starved**	**Test Statistic**	***P* Value**			**Test Statistic**	***P* Value**
Carbohydrate metabolism: *glycolysis/gluconeogenesis*									
Glucose-6P isomerase	*pgi*	0.089 (0.006)	0.156 (0.008)	48.565	<0.001*,[a]	0.083 (0.007)	0.078 (0.006)	0.28	0.605
6-phophofructo-2-kinase	*pfk*	0.053 (0.005)	0.079 (0.007)	11.644	0.003*,[a]	0.052 (0.005)	0.066 (0.007)	3.015	0.104
Phosphoglycerate mutase	*pgm*	0.028 (0.002)	0.053 (0.003)	39.931	<0.001*,[a]	0.024 (0.003)	0.024 (0.002)	0.008	0.93
Enolase	*eno*	0.042 (0.006)	0.063 (0.008)	3.499	0.075	0.048 (0.014)	0.036 (0.004)	0.003	0.957
Pyruvate kinase	*pyk*	0.465 (0.046)	0.588 (0.039)	4.219	0.053	0.407 (0.029)	0.360 (0.043)	0.891	0.361
Phosphoenolpyruvate carboxykinase	*pepck*	0.029 (0.007)	0.089 (0.009)	40.397	<0.001*,[a]	0.043 (0.027)	0.157 (0.041)	57	0.007*,[a]
Pyruvate carboxylase subunit A	*pc*	0.005 (0.001)	0.007 (0.001)	2.047	0.167	0.003 (9.1^{-4})	0.005 (0.002)	1.104	0.311
Glycogen									
UDP-glucose pyrophosphorylase	*ugpase*	0.028 (0.003)	0.044 (0.004)	10.618	0.004*,[a]	0.026 (0.003)	0.018 (0.002)	8	0.013
Pentose phosphate pathway									
Glucose-6P dehydrogenase	*g6pd*	0.002 ($1.1\ E^{-4}$)	0.003 ($2.0\ E^{-4}$)	17.066	<0.001*,[a]	0.001 ($1.4\ E^{-4}$)	0.001 ($5.1\ E^{-5}$)	18	0.153
TCA cycle									
Citrate synthase	*cs*	0.002 ($2.4E^{-4}$)	0.002 ($2.7\ E^{-4}$)	105	0.016*,[a]	9.7^{-4} ($2.5\ E^{-4}$)	9.1 E^{-4} ($1.9\ E^{-4}$)	0.029	0.867
Acetyl-CoA									
Dihydrolipoamide acetyltransferase component of Pyruvate dehydrogenase	*dlat*	0.044 (0.005)	0.048 (0.003)	0.46	0.505	0.039 (0.004)	0.037 (0.004)	0.094	0.764
Acetyl-CoA synthetase	*accoas*	0.010 (0.002)	0.009 (0.002)	0.258	0.617	0.009 (0.002)	0.005 (0.001)	2.668	0.125
Fatty acid metabolism									
ATP citrate lyase	*atpcl*	0.105 (0.078)	0.056 (0.007)	23.167	<0.001*,[b]	0.059 (0.003)	0.038 (0.007)	16.656	0.002*,[b]
Acetyl-CoA carboxylase	*acc*	0.032 (0.004)	0.028 (0.004)	0.665	0.424	0.025 (0.002)	0.017 (0.002)	6.534	0.023
Fatty acid synthase	*fas*	0.006 (0.001)	0.004 (0.001)	3.955	0.06	0.003 ($3.1\ E^{-4}$)	0.002 ($3.5\ E^{-4}$)	0.932	0.351
Stearoyl CoA desaturase	*scd*	$4.5\ E^{-5}$ ($4.4\ E^{-6}$)	$3.7\ E^{-5}$ ($4.8\ E^{-6}$)	1.155	0.295	$2.6\ E^{-5}$ ($5.0\ E^{-6}$)	$1.9\ E^{-5}$ ($3.3\ E^{-6}$)	1.083	0.316
Long-chain fatty acyl CoA synthetase	*lcfacs*	$4.3\ E^{-4}$ ($5.2\ E^{-5}$)	$8.6\ E^{-4}$ ($1.2\ E^{-4}$)	14.33	0.001*,[a]	$4.1\ E^{-4}$ ($3.4\ E^{-5}$)	$4.5\ E^{-4}$ ($8.6\ E^{-5}$)	0.318	0.582
Glycerolipid metabolism: *glycerol*									
Glycerol-3P dehydrogenase	*gpdh*	0.234 (0.016)	0.247 (0.015)	0.349	0.561	0.206 (0.015)	0.135 (0.014)	11.739	0.004*,[b]
Glycerol kinase	*gk*	$1.7\ E^{-4}$ ($2.1\ E^{-5}$)	$2.0\ E^{-4}$ ($3.6\ E^{-5}$)	78	0.460	$1.2\ E^{-4}$ ($3.1\ E^{-5}$)	$1.3\ E^{-4}$ ($2.1\ E^{-5}$)	40	0.368
Glycerolipids									
1-acyl-sn-glycerol-3-phophate acyltransferase	*agpat*	0.014 (0.001)	0.015 (0.002)	0.198	0.661	0.011 ($7.9\ E^{-4}$)	0.006 ($9.8\ E^{-4}$)	11.955	0.004*,[b]
Diacylglycerol o-acyltransferase	*dgat*	0.006 (0.001)	0.005 (0.001)	0.63	0.436	0.005 ($4.8\ E^{-4}$)	0.004 ($3.2\ E^{-4}$)	3.786	0.072
Glycerol-3P o-acyltransferase	*gpat*	$1.4\ E^{-4}$ ($2.0\ E^{-5}$)	$1.9\ E^{-4}$ ($3.7\ E^{-5}$)	1.177	0.29	$1.7\ E^{-4}$ ($2.0\ E^{-5}$)	$1.6\ E^{-4}$ ($2.2\ E^{-5)}$	25	0.491
Phosphatidate phosphatase	*pp*	0.004 ($4.6\ E^{-4}$)	0.005 ($4.5\ E^{-4}$)	83	0.295	0.005 ($5.3\ E^{-4}$)	0.003 ($3.4\ E^{-4}$)	7.863	0.014
Phospholipase d	*pld*	$3.9\ E^{-4}$ ($4.4\ E^{-5}$)	$5.1\ E^{-4}$ ($5.5\ E^{-5}$)	2.661	0.118	$4.8\ E^{-4}$ ($7.7\ E^{-5}$)	$3.4\ E^{-4}$ ($5.1\ E^{-5}$)	2.387	0.145
Triacylglycerol lipase	*tgl*	0.008 ($7.3\ E^{-4}$)	0.011 ($7.6\ E^{-4}$)	97	0.056	0.008 ($8.7\ E^{-4}$)	0.006 ($5.4\ E^{-4}$)	4.526	0.052
Other pathways									
AMP-activated protein kinase	*ampk*	0.001 ($8.7\ E^{-5}$)	0.001 ($6.4\ E^{-5}$)	2.897	0.103	$6.9\ E^{-4}$ ($1.2\ E^{-4}$)	$6.8\ E^{-4}$ ($1.2\ E^{-4}$)	0.006	0.942
cGMP-dependent protein kinase	*pkg*	0.001 ($1.4\ E^{-4}$)	0.002 ($3.2\ E^{-4}$)	9.809	0.005*,[a]	0.001 ($1.2\ E^{-4}$)	0.001 ($1.8\ E^{-4}$)	0.199	0.662
Lipid storage droplet 2	*lsd2*	0.197 (0.022)	0.175 (0.008)	48	0.268	0.142 (0.012)	0.134 (0.010)	0.282	0.603

*Indicates differences are significant after correction for multiple testing.

[a]Genes that are significantly down-regulated in fed compared with starved females.

[b]Genes that are significantly up-regulated in fed compared with starved females.

showed decreased transcriptional activity in fed females, phosphoenolpyruvate carboxykinase (*pepck*) and citrate synthase (*cs*). *Pepck* codes for a critical irreversible enzyme in gluconeogenesis, wherein unfed individuals are creating glucose *de novo* from other substrates to meet the needs of critical tissues like the brain. Citrate synthase performs the first step of the TCA cycle and down-regulation of the TCA cycle is consistent with reduced energy metabolism during a starvation response (fig. 1).

The only lipid synthetic gene that showed increased transcription levels after short-term sugar feeding was ATP citrate lyase (*atpcl*), which catalyzes the release of cytosolic acetyl-CoA, as a precursor for fatty acid synthesis (fig. 1). Yet, none of the key genes involved in fatty acid synthesis (*acc* and *fas*) showed a significant increase in transcript abundance in fed females (table 2). Although *fas* transcript abundance was 1.5-fold higher in the fed treatments, this change was not significant. The metabolic fate of the released cytosolic acetyl-CoA therefore, remains unclear, but acetyl-CoA was not incorporated into fatty acids as evidenced by the lack of deuterium incorporation in our isotopic-tracking experiment.

Long-term feeding led to differences in transcript abundance for 4 out of 28 genes when comparing fed to starved *N. vitripennis* females (fig. 1 and table 2). As in the short-term feeding treatment, no significant induction of *acc* or *fas* transcription was observed after feeding and constitutive *fas* gene transcription was reduced to trace levels. Again, this is consistent with fatty acid synthesis being impaired in *N. vitripennis*.

Two of the four differentially transcribed genes in the long-term feeding treatment were also found to respond significantly in short-term fed females. Phosphoenolpyruvate carboxykinase (*pepck*) had lower transcript abundance in sugar-fed wasps suggesting lower gluconeogenesis in fed animals, whereas ATP citrate lyase (*atpcl*) had higher transcript abundance under fed conditions (fig. 1). Two genes that did not significantly respond in the short-term fed treatment had greater transcript abundance after long-term feeding: glycerol-3P dehydrogenase (*gpdh*), and 1-acyl-sn-glycerol-3P acyltransferase (*agpat*). The former is essential for glycerol synthesis, an important component of di- and triglycerides, the latter catalyzes the reaction that converts acylglycerol-3P to diacylglycerol-1P for use in glycerolipid components of cell membranes. The response of these genes could indicate a redistribution of lipids, for example, phospholipids may still be synthesized from triglyceride stores and be used to maintain the integrity of cell membranes.

Discussion

With the exception of studies on endosymbionts (Dale and Moran 2006; Maughan et al. 2007) and cavefish (Jeffery 2009), the importance of changes in transcriptional regulation as a cause of trait regression have not been well studied. Particularly, little is known about mechanisms underlying trait degradation in animals, even though many species show phenotypic degradation of unwanted or unused traits, as well as traits that are still required for successful growth, survival, and reproduction (Ellers et al. 2012). The parasitoid wasp *N. vitripennis* provides an excellent system to do so, given the recent completion of its genome sequence (Werren et al. 2010). Our results showed lack of transcriptional regulation of key genes in lipid synthesis after short- and long-term sugar feeding, providing a potential molecular mechanism for the observed lack of lipid synthesis in *N. vitripennis*.

Physiological and Transcriptional Responses to Feeding

The results of the feeding experiment showed lack of lipid accumulation after sugar feeding, as is typical for parasitoid wasps (Ellers 1996; Rivero and West 2002; Visser et al. 2010). Although sugar feeding allowed the females to economize on their lipid use and slowed the rate of lipid depletion, compared with females under starved conditions, no isotopic labels were recovered in the lipid fraction when *N. vitripennis* females were fed deuterated sugar solution. This is in sharp contrast with our findings in the honeybee *A. mellifera*, which readily incorporated isotopic labels after sugar feeding, and which is known to synthesize lipids when sugar-fed (Hasegawa et al. 2009). Together, these findings provide strong evidence that *N. vitripennis* does not synthesize fatty acids when fed sugars, neither to accumulate lipids nor to balance an increased catabolism of fatty acids.

Consistent with the biochemical data, our estimates of transcript abundance of lipid synthetic genes indicate that fatty acid synthesis is not occurring in sugar-fed wasps. We found no effect of sugar feeding on the transcription levels for *acc* and *fas*, the two key genes for which an active gene transcription is crucial for fatty acid synthesis. *Fas*, which encodes the enzyme that performs the majority of steps involved in fatty acid synthesis, only showed trace levels of transcripts in *N. vitripennis*, regardless of nutritional status. These data provide strong evidence for transcriptional regulation underlying lack of active lipid synthesis in *N. vitripennis*.

Further support is provided by the lower transcript abundance of glucose-6P dehydrogenase (*g6pd*) of the pentose–phosphate pathway in fed, compared with starved wasps. *G6pd* produces the reducing agent of nicotinamide adenine dinucleotide phosphate (NADP), NADPH, and should typically increase after feeding to generate higher NADPH levels for use in fatty acid synthesis (Salati and Amir-Ahmady 2001). The lack of transcriptional response of *g6pd* to sugar feeding in *N. vitripennis* suggests that there was no increase in the production of NADPH through the pentose–phosphate pathway, in line with the unresponsiveness of fatty acid synthesis genes.

Starvation, on the other hand, resulted in transcriptional responses that were largely consistent with those observed

in other animals (Kersten 2001; Duplus and Forest 2002). We found that the majority of genes involved in carbohydrate metabolism and TCA cycle were transcribed at a higher rate in starved females. Starvation causes an acute shortage of glucose, which typically leads to an activation of the gluconeogenesis pathway. The observed increase in transcription of *pepck* plays a pivotal role in this response by catalyzing a rate-limiting step for converting pyruvate to glucose (Reshef et al. 2003). Also, *pepck* is involved in glyceroneogenesis, an alternative pathway that produces glycerides from pyruvate instead of glucose (fig. 1).

In addition, starvation requires catabolism of triglycerides to increase the availability of free fatty acids for β-oxidation to release energy and to maintain cell membrane lipids. Indeed, lipid levels of starved *N. vitripennis* females decreased sharply over time (fig. 2). Also, we found higher transcript abundance of genes involved in lipolysis, such as long-chain fatty acyl CoA synthetase (*lcfacs)* during starvation, suggesting that fatty acids are broken down during β-oxidation, releasing acetyl-CoA for use in gluconeogenesis. To restrain loss of free fatty acids during lipolysis, glyceroneogenesis allows re-esterification of fatty acids (Reshef et al. 1970, 2003). An increase in levels of unsaturated and long-chain fatty acids can therefore, act as signals involved in gene regulation, for instance by stimulating transcription of *pepck* (Duplus and Forest 2002).

Also, cGMP-dependent protein kinase (*pkg*) transcription was higher in starved females. This gene is involved in phosphorylation of protein substrates that stimulate lipolysis (Holm et al. 2000). Furthermore, increased transcription of *pkg* has been associated with starvation, and increased food searching behavior in several species of insects, including other hymenopterans like honeybees and ants (Kaun and Sokolowski 2009). Our results for *N. vitripennis* females under starvation thus conform to findings in other animal species, in which gluconeogenesis is activated to increase glucose levels and free fatty acids are burned or re-used for the formation of other lipid types.

Evolutionary Changes in Lipogenic Regulatory Mechanisms

Although the transcriptional response to sugar feeding has been extensively studied in vertebrates (Abu-Elheiga et al. 2001, 2005; Duplus and Forest 2002), few such studies have been carried out in insects. The few data available, however, support our findings that changes in transcriptional regulation of key lipogenic genes underlie the lack of lipid synthesis in *N. vitripennis*. When we reanalyzed data of Zinke et al. (2002) to directly compare short-term sugar-fed with starved *Drosophila melanogaster* larvae, *fas* expression was significantly higher in fed larvae than in starved larvae, contrasting the lack of response we observed in *N. vitripennis*.

In another *D. melanogaster* study, re-feeding of adult females after starvation caused increased transcription of fatty acid synthesis genes and lowered transcript levels of genes involved in fatty acid oxidation (Gershman et al. 2007). Whereas *cs* and *g6pd* were up-regulated in re-fed *D. melanogaster*, these genes were down-regulated under fed conditions in *N. vitripennis*. Increased expression of these genes leads to the production of NADPH used in fatty acid synthesis and citrate, composing the first step in the TCA cycle. Overall, transcriptional patterns observed in *N. vitripennis* indicate that fatty acids are oxidized, rather than synthesized under the fed condition, opposing findings in *D. melanogaster* that readily synthesizes lipids when fed (Geer et al. 1985) .

Despite the lack of induced gene transcription of *fas* and *acc* in fed compared with starved females, under both conditions, we detected transcripts for these genes, albeit for *fas* transcript levels were very low. Why then is lipogenesis not active in *N. vitripennis*? First, low transcript persistence of *fas* in *N. vitripennis* could be the result of directed gene transcription, initiated from the *fas* promoter. However, transcription might not be sufficient to generate substantial amounts of enzyme necessary for a fully functional fatty acid synthesis pathway. Second, low transcript levels of *fas* might be the result of the physical positioning of the gene. In *D. melanogaster*, highly transcribed genes are known to affect the rate of transcription of genes in close proximity (Spellman and Rubin 2002). The majority of genes neighboring *fas* in *N. vitripennis* have various functions, including an ATPase AAA family protein, a splicing factor, a purine nucleoside phosphorylase, and a ubiquitin activating protein. Read-through of RNA polymerase actively transcribing genes nearby *fas* could have resulted in the leaky low transcript levels that we observe in our presented qPCR assays. Third, gene expression can be highly tissue-specific (Whitehead and Crawford 2005). Most processes associated with lipid metabolism are expected to occur at the highest rate within the fat body, a diffusely structured organ filling a major part of the abdominal cavity. We used whole bodies to extract RNA, mainly because extracting an intact fat body is notoriously difficult in parasitoids. However, overall transcript levels could be higher when solely inspecting the fat body, which could be accomplished by using female abdomens rather than whole bodies.

Lack of lipogenesis could also be associated with mutation accumulation in the structural parts of the genes *acc* or *fas* or their truncation within the genome. However, full-length *fas* and *acc* sequences are present in the genome of *N. vitripennis*, and alignment of these sequences with homologs from other hymenopteran species revealed no signs of degradation. Although enzyme assays are needed to confirm functionality, there are no indications that mutations have accumulated in the structural part of these genes in *N. vitripennis*. Alternatively, impaired enzyme functioning could be due to other factors that optimize enzyme activity, such as sufficient

availability of enzymatic co-factors (Shakoury-Elizeh et al. 2010). Moreover, the rate of metabolomic responses typically depends only partly on gene transcription and can also be affected by substrate or product levels and the physiological status within certain tissues (ter Kuile and Westerhoff 2001; Iizuka et al. 2004; Suarez and Moyes 2012).

The most plausible explanation for unresponsiveness of key lipogenic genes to food in *N. vitripennis* is regulatory inhibition of gene transcription. Noncoding promoter regions are not under purifying selection and typically the rate of mutation is increased in these regions (Stone and Wray 2001; Wray et al. 2003). In rats, *fas* contains both, a relatively small promoter region that increases *fas* transcription 2- to 3-fold in response to insulin, and a larger promoter region located 6,000 bp upstream of *fas,* which contains a carbohydrate responsive element that increases transcription of *fas* 20- to 30-fold when glucose is available (Rufo et al. 2001). Mutations accumulated in these regions of the *fas* promoter could impair lipogenic ability in parasitoids. Alternatively, mutations in genes encoding transcription factors may prohibit lipogenesis. For example, mutant *D. melanogaster* larvae that lack the homolog of sterol regulatory element binding protein (SREBP), an essential transcription factor for cholesterol and unsaturated fatty acid synthesis, exhibited pronounced growth defects and died prematurely due to improper gene regulation (Kunte et al. 2006). Identification and testing the functionality of genes underlying essential transcription factors for successful functioning of *fas* in *N. vitripennis* could reveal if the unresponsiveness of *fas* is due to mutation in genes encoding essential transcription factors.

Irrespective of the exact molecular mechanism that has led to transcriptional unresponsiveness of lipogenic genes to sugars, a lack of transcription would be expected to lead in time to associated accumulation of mutations in the coding part of the genes that are not transcriptionally active. Lack of lipogenesis is thought to have originated in the basal parasitic Hymenoptera, with the first parasitic groups appearing in the fossil record during the early Jurassic (180–200 Ma) (Quicke 1997; Visser et al. 2010). Even when conservative mutation rates are considered, unused genes would be expected to have accumulated mutations. The fact that such degradation of the *fas* gene has not occurred in *N. vitripennis* is remarkable and can only be explained if there is a selective advantage to maintain functionality of *fas,* although the nature of such benefit remains speculative. Lipogenesis re-evolved in at least three parasitic wasp lineages (Visser et al. 2010), mainly in species that are expected to lack active physiological manipulation of their hosts. However, functional lipogenesis has also been reported in different populations of the same species under varying environmental conditions (Moiroux et al. 2010). The ease with which lipogenesis is restored is thus consistent with the absence of mutation accumulation in the coding part of *fas* and suggests altered regulation of gene expression to underlie phenotypic regression of this essential trait in parasitoids.

Supplementary Material

Supplementary table 1 is available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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